LIGHT-INDUCED HINDRANCE OF ROTATIONAL MOTION OF THE SPIN LABEL TEMPAMINE IN THE AQUEOUS LUMEN OF SPINACH THYLAKOIDS

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1. Introduction

Electron paramagnetic resonance (EPR) spectroscopy has been developed to measure internal viscosity, volume and pH in membrane-surrounded aqueous regions of cells and organelles [1–9]. In the viscosity studies, the motion of a spin label located in a membrane bound aqueous space is hindered relative to the motion of the spin label in bulk water. Thus, the rotational motion of 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amine (Tempamine) has been shown restricted by a factor of ~7 when residing in the lumen of thylakoids in the dark [7,8]. However, experiments requiring light intensities which can support photophosphorylation are problematic since the thylakoids rapidly photoreduce nitroxyl radicals [7,8,10–13] to the corresponding diamagnetic hydroxylamines [13]. Thus there are no reports of the motion of spin labels residing in the lumen of light-activated thylakoids available to date.

Here we report a light-induced change in the rotational motion of Tempamine located in the lumen of spinach thylakoids, and we discuss how this change may be related to light-induced membrane conformational changes [14–21].

2. Materials and methods

Tempamine was purchased from Aldrich (Milwaukee, WI) and used without further purification. Tris(oxalato)chromate (III), (chromium oxalate) was synthesized by the methods in [22] and characterized by its visible spectrum [23] and EPR *g*-value [24–26].

EPR spectra were recorded on a Varian E-9 spectrometer using an E-231 cavity with the irradiation grid plate removed to allow actinic illumination. White light from a 500 W projector was heat-filtered and focused through a 16 liter Florence flask containing 5 mM $CuSO_4$. The light intensity applied to the cavity was ~400 kergs . s^{-1} . cm^{-2}, 50% of which is transmitted through the grid. Rotational correlation times (τ_c) were calculated from the equation [27–29]:

$$\tau_c = kW_0\,([h_0/h_1]^{1/2}-1)$$

where k is a constant which depends on the principal values of g and the hyperfine tensors for the spin label and on the microwave frequency. We use the empirically determined value $k = 6.5 \times 10^{-10}$ since the appropriate crystal parameters for Tempamine are not known. The apparent peak-to-peak midfield line width, midfield line height and highfield line height are W_0, h_0, and h_{-1}, respectively, and are measured from the first derivative spectra. EPR signals arising from Tempamine located only in the aqueous lumen of the thylakoid were observed with the techniques detailed in [7], but using chromium oxalate, a more effective broadening agent [8].

Thylakoids were isolated from fresh market *Spinacea oleracea* as in [7]. Chlorophyll concentrations were determined as in [30]. All isolation procedures were at <4°C.

3. Results

Fig.1A shows the EPR tracing obtained at a constant magnetic field which monitors the positive peak of the midfield absorption in the first derivative spectrum. When the sample is illuminated there is a rapid

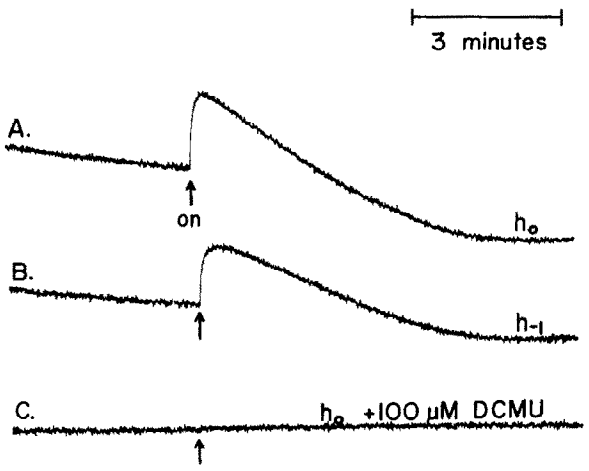

Fig.1. Light-induced changes in signal height with time. (A) EPR tracing of a sample containing 3 mM Tempamine (pH 7.5), 40 mM chromium oxalate (pH 7.5), 5 mM sodium phosphate (pH 7.5), 1 mM ADP, 0.25 mM methyl viologen, and chloroplasts at 5 mg chl/ml. The magnetic field is constant at 3242.2 G, which corresponds to the midfield line (h_0). EPR instrument settings: gain, 6.3 × 10^3; modulation, 0.50 G; time constant, 0.128 s. The onset of illumination is indicated by the arrow. (B) Same as in (A) except the magnetic field is constant at 3259.2 G, which corresponds to the highfield line (h_{-1}). (C) Same as in (A) with the addition of 100 μM DCMU to the sample.

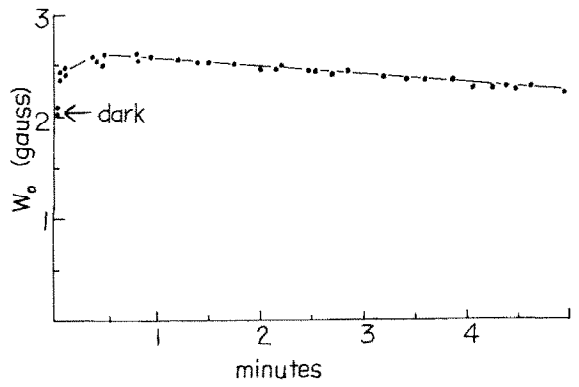

Fig.2. Light-induced changes in midfield line width with time. Samples are as in fig.1A. Scan rate was 20 G/min. The arrow indicates the line width from a sample maintained in the dark.

increase in signal amplitude followed by a slower decrease for ~5 min. This same effect is seen in fig.1B when the magnetic field is adjusted to monitor the highfield peak. When no Tempamine is added to an otherwise complete reaction mixture, no signal of any kind is observed. The addition 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) prevents the light-induced signal increase as shown in fig.1C but it does not affect the magnitude of the signal in the dark prior to illumination.

The tracings in fig.1A,B also show that the decrease in signal amplitude is linear between ~20 s and 4.5 min illumination. When the midfield line width is plotted against time as in fig.2, a light-induced increase is followed by a slower decrease in W_0. The decrease is linear after ~30 s illumination. Since the total time required for a forward/reverse scan of a sample is 4 min, it is possible to average the values obtained for W_0, h_0, and h_{-1} and to calculate τ_c, as long as the scans of the mid- and highfield lines are recorded over the period of linear change.

Fig.3 (top) shows typical forward/reverse spectra obtained from thylakoids maintained in the dark. The small decrease in signal amplitude with time does not appreciably affect the calculation of τ_c as seen in table 1. The bottom spectra in fig.3 shows that upon illumination, the signal rapidly decreases. Since a scan takes 2 min, the midfield line is scanned after ~1 min illumination and it is rescanned (in reverse) after ~3 min illumination. The highfield line is also scanned twice during 1 and 3 min. Thus, both the mid- and highfield lines are scanned during the period of linear reduction and the averaging technique may be applied. The average values for W_0, h_0, and h_{-1} are given in table 1 and are used to calculate τ_c. These values give a close approximation of the actual τ_c of Tempamine in the illuminated thylakoid. Table 1 shows that the rotational motion of Tempamine is restricted by a factor of ~7.4 in the dark thylakoid lumen when compared with the motion of Tempamine in bulk water. However, Tempamine motion is restricted by a factor of ~11.6 in the illuminated thylakoid lumen. Thus, illumination imposes an additional 57% hindrance on the rotation of Tempamine in the lumen.

4. Discussion

The light-induced increase in signal amplitude observed in fig.1A,B is most likely due to the rapid uptake of the amine spin label from the exterior of the thylakoids in response to the light-driven internal acidification of the lumen [31,32]. Since the lumen contains no broadening agent [7,8] the observed signal increases as the Tempamine in the lumen is joined

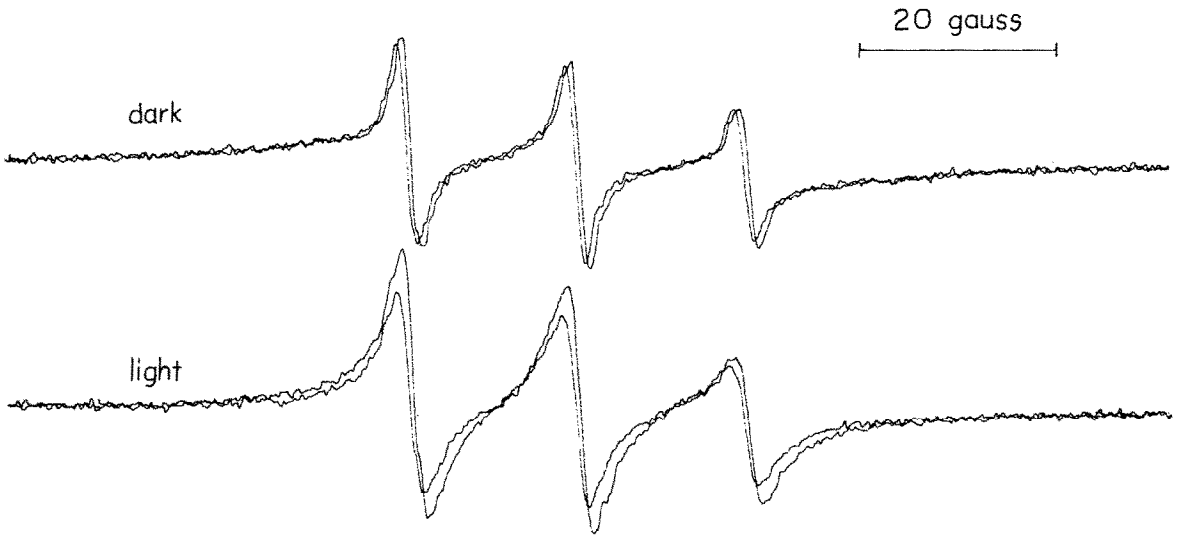

Fig.3. Forward and reverse EPR spectra of thylakoids in the dark (top) and during illumination (bottom). Sample and EPR settings are as in fig.1A, except the scan rate is 100 G/min.

by additional influxing Tempamine. The slow decay in signal amplitude is the photoreduction of the Tempamine nitroxyl to the corresponding hydroxylamine [13], also observed in [10–12]. Fig.1C shows that the light-induced changes are dependent on electron transport, since DCMU is a potent inhibitor of electron transport.

The motion of Tempamine in the illuminated thylakoid lumen is restricted relative to the motion of Tempamine in the lumen of dark thylakoids as shown in table 1. The hyperfine coupling constant indicates that the Tempamine remains in a highly polar environment even after illumination. The relationship between our results and the light-induced conformational changes reported by others is not altogether clear. The light-induced changes in absorbance and light scattering in [14–17] are probably manifestations of thylakoid swelling and shrinkage. The phenomenon of shrinkage is most clearly visualized in [18,19] where, in response to light, the distance between the thylakoid membranes is shown reduced and the membranes themselves become thinner. If this is occurring in our illuminated thylakoids, then all the water in the lumen is closer to the membrane. Membranes may play an important role in ordering water within cells [1,2,9]. Thus, reducing the space between the thyla-

Table 1
Summary of parameters measured and calculated from EPR spectra

	Conditions	h_0 (cm)	h_{-1} (cm)	h_0/h_{-1}	W_0 (G)	A_n (G)	τ_c (s)	η
Water	–	11.6	10.8	1.07	1.7	16.9	0.40×10^{-10}	1.0
Thylakoids	Dark, forward	4.14	2.80	1.47	2.1	16.7	2.90×10^{-10}	7.25
	Dark, reverse	3.96	2.66	1.49	2.1	16.7	3.01×10^{-10}	7.53
	Dark, average	4.05	2.73	1.48	2.1	16.7	2.96×10^{-10}	7.40
Thylakoids	Light, forward	4.94	2.92	1.69	2.6	16.5	5.07×10^{-10}	12.7
	Light, reverse	3.84	2.40	1.60	2.4	16.5	4.13×10^{-10}	10.3
	Light, average	4.39	2.66	1.65	2.5	16.5	4.62×10^{-10}	11.6

The water sample contained only Tempamine (pH 7.5) at 3 mM. The thylakoid samples are as described in fig.1A, as are the EPR settings. Scan rate was 20 G/min

$\eta = \tau_c$ (sample)/τ_c (water)

koid membranes may increase the total internal water order and consequently increase the measurement of τ_c in the lumen. Since protons are also known to increase water order [33], the acidification of the lumen [6] may place additional hindrance on the rotational freedom of Tempamine. On the contrary, Tempamine, being an amine, may tend to reduce water order [34]. However, the net effect of all of these contributions as shown by this study is increased hindrance of Tempamine motion in the thylakoid lumen.

Other studies of light-induced conformational changes demonstrate an increase in diazoniumbenzene sulfonate labeling of thylakoid membranes [20,21] and a decrease in the ability of a water-soluble carbodiimide to inhibit calcium binding of thylakoids [35]. Since diazoniumbenzene sulfonate is membrane impermeable and since calcium binding occurs on the stroma side of the thylakoid, both of these techniques measure events at the external surface of the thylakoid and do not necessarily report changes at the lumen side of the thylakoid. Thus our findings do not seem to be directly related to these studies.

Acknowledgements

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